Production of fructooligosaccharides by mycelium-bound transfructosylation activity present in *Cladosporium cladosporioides* and *Penicilium sizovae*

P. Zambelli[a], L. Fernandez-Arrojo[b], D. Romano[a], P. Santos-Moriano[b], M. Gimeno-Perez[c] , A. Poveda[d], R. Gandolfi[e], M. Fernández-Lobato[c], F. Molinari[a] and F.J. Plou[b,*]

[a] *Department of Food Environmental and Nutritional Science (DeFENS), University of Milan, 20133 Milan, Italy*

[b] *Instituto de Catalisis y Petroleoquimica, CSIC, 28049 Madrid, Spain*

[c] *Centro de Biologia Molecular Severo Ochoa, UAM-CSIC, 28049 Madrid, Spain*

[d] *Servicio Interdepartamental de Investigacion, UAM, 28049 Madrid, Spain*

[e] *Department of Pharmaceutical Science, University of Milan, 20133 Milan, Italy*

*Corresponding author: Francisco J. Plou, Instituto de Catalisis y Petroleoquimica, CSIC, Marie Curie 2, 28049 Madrid, Spain. Phone: +34-91-5854869. Fax: +34-91-5854760. E-mail: fplou@icp.csic.es.

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ABSTRACT

Different filamentous fungi isolated from molasses and jams (kiwi and fig) were screened for fructooligosaccharides (FOS) producing activity. Two strains, identified as *Penicilium sizovae* (CK1) and *Cladosporium cladosporioides* ($CF_2$15), were selected on the basis of the FOS yield and kestose/nystose ratio. In both strains the activity was mostly mycelium-bound. Starting from 600 g/L of sucrose, maximum FOS yield was 184 and 339 g/L for *P. sizovae* and *C. cladosporioides*, respectively. Interestingly, the highest FOS concentration with *C. cladosporioides* was reached at 93% sucrose conversion, which indicated a notable transglycosylation to hydrolysis ratio. The main FOS in the reaction mixtures were identified by HPAEC-PAD chromatography. *C. cladosporioides* synthesized mainly 1-kestose (158 g/L), nystose (97 g/L), ^{1}F-fructosylnystose (19 g/L), 6-kestose (12 g/L), neokestose (10 g/L) and a disaccharide (34 g/L) that after its purification and NMR analysis was identified as blastose [Fru-β(2→6)-Glc]. *P. sizovae* was very selective for the formation of ^{1}F-FOS (in particular 1-kestose) with minor contribution of neoFOS and negligible of levan-type FOS.

Key words: Fructooligosaccharides; transfructosylation; prebiotics; bioactive oligosaccharides; blastose;

1. INTRODUCTION

Inulin-type fructooligosaccharides (^{1}F-FOS) are fructose oligomers with a terminal glucose unit in which 2-4 fructofuranosyl moieties are linked by β(2→1) bonds [1;2]. ^{1}F-FOS are used as food ingredients due to their properties, such as prebiotic action favouring the development of bifidobacteria and lactobacillus, low caloric intake (2 kcal/g), low glycemic index, improved gut absorption of Ca^{2+} and Mg^{2+}, lowering of blood lipid levels, prevention of urogenital infections and reduced risk of colon cancer [3;4].

^{1}F-FOS are commonly obtained by controlled hydrolysis of inulin or other fructans (typically using inulinases, EC 3.2.1.7) [5] or by enzymatic transfructosylation of sucrose catalyzed by β-fructofuranosidases (EC 3.2.1.6) or fructosyltransferases (EC 2.4.1.9) [6]. Short-chain ^{1}F-FOS (1-kestose, nystose, 1^{F}-fructosylnystose, and so on) are currently produced at multi-ton scale from concentrated sucrose solutions using fungal transfructosylating enzymes from *Aspergillus niger*, *Aspergillus oryzae* or *Aureobasidium pullulans* [7;8].

Other FOS containing β(2→6) linkages between two fructose units (^{6}F-FOS, also called levan-type FOS, such as 6-kestose or 6-nystose) [9-12] or between a fructose and a glucose (^{6}G-FOS, also called neoFOS, such as neokestose or neonystose) have also been described and are commonly produced by enzymes from yeasts [13-15]. Levan-type FOS and neoFOS are reported to exhibit improved prebiotic properties and chemical stability compared to inulin-type FOS [16-18],

1 although more studies are required to elucidate the bioactivity of the different FOS
2 series.

3 Industrial scale production of FOS is performed by either soluble enzymes in
4 batch reactions [19] or by entrapped cells in alginate gel beads using continuous
5 fixed-bed reactors [20;21]. Several immobilized enzymes for FOS synthesis at
6 laboratory scale have also been reported [6;22-24]. The use of biomass (e.g. mycelia)
7 or immobilized biocatalysts minimizes the loss of activity during operation and
8 allows establishing a continuous process.

9 The identification of novel microbial strains with high transfructosylation
10 activity and/or producing a distinctive FOS pattern is currently being investigated
11 [25]. In this work, we have screened different microorganisms able to grow on
12 sucrose-rich substrates such as molasses or jams with the aim of identifying new
13 transfructosylating enzymes able to produce FOS with different composition
14 compared with already known biocatalysts.
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2. MATERIALS AND METHODS

2.1. Materials

Sucrose, glucose, fructose and *p*-anisaldehyde were from Sigma-Aldrich. Nystose and 1-kestose were from Fluka. 1^F-fructosylnystose was from Megazyme. 6-Kestose, neokestose and neonystose were synthesized as previously described [9;13;14]. Yeast extract was from Difco and barley malt flour from Diagermal. All other reagents and solvents were of the highest available purity and used as purchased.

2.2. Isolation and screening of microorganisms with transfructosylating activity

The fungal cultures employed in the study were isolated from molasses or from commercial and home-made kiwi and fig jams. The contents of each sample were uniformly mixed, and a sample (1.0 mL) was aseptically withdrawn, mixed with 9.0 ml of sterile water, and then diluted for isolation purposes. The inoculum was transferred to Potato Dextrose Agar (PDA) plates containing chloramphenicol (0.05 g/L) to inhibit bacterial growth. Plates were repeatedly incubated at 28°C until obtaining homogenous morphological colonies. Identification of the best performing strains was carried out at CBS (Centraal Bureau voor Schimmelcultures, Baarn, Holland) using standard molecular techniques. The microorganisms were routinely maintained on MYA slants (Barley malt flour 100 g/L, yeast extract 5 g/L, agar 15 g/L, pH 5.5) at 4°C.

The screening for FOS production was performed by inoculating the strains into 1 L flasks containing 100 mL of liquid MY medium (Barley malt flour 100 g/L,

yeast extract 5 g/L, pH 5.5) containing 200 g/L of sucrose. The growth was carried out on a reciprocal shaker (150 rpm) at 28°C. Sugar composition was analyzed by HPLC during the growth after filtration of the mycelium.

Studies were carried out with lyophilized mycelium obtained after growth on MY medium with and without 200 g/L sucrose) for different times under the conditions described above. Mycelium with the highest activity was found when cultures were performed using MY medium without 200 g/L sucrose after 96 h on a reciprocal shaker (150 rpm) at 28°C. After centrifugation, cells were washed with sodium acetate buffer (20 mM, pH 6.0), lyophilized and used for biotransformations.

2.3. Biotransformations with *Cladosporium cladosporioides* and *Penicillium sizovae*

The activity of mycelium-bound and extracellular enzymes was independently assayed [26]. Biotransformations aimed at FOS production were carried out with growing, re-suspended and lyophilized mycelium and with mycelium-free supernatant of *Cladosporium cladosporioides* and *Penicillium sizovae*. In experiments with whole growing cultures, the substrate was added together with the medium and biotransformation followed during the growth. Experiments with mycelium-free supernatant were accomplished using the liquid fraction obtained after centrifugation of the whole culture and ultrafiltration using a stirred ultrafiltration cell (Model 8050 Amicon, Millipore, capacity 50 ml) with a 10.000 Da cut-off membrane. The extracellular fraction containing 0.41 g/L of total protein (Bradford assay) was used for biotransformation, started by incubating the mixture at 50°C in an orbital shaker at 90 rpm after addition of 200 g/L of sucrose. Freshly

suspended (40 $g_{dry\ weight}$/L) and lyophilized mycelium (40 g/L) were added to a 200-600 g/L sucrose solution in 20 mM sodium acetate (pH 6.0) in a total reaction volume of 2 mL. The mixture was incubated at 50ºC in an orbital shaker at 90 rpm. At different times, aliquots (50 µL) were withdrawn, diluted with 200 µL of water, incubated for 10 min at 90ºC to inactivate the enzymes, and analysed by HPLC to determine the total FOS yield and by HPAEC-PAD to identify the synthesized FOS.

2.4. HPLC analysis

The screening of transfructosylation activity and the measurement of the FOS production were carried out analyzing the corresponding reaction mixtures using hydrophilic interaction chromatography (HPLC-HILIC) with a Delta 600 quaternary pump (Waters). The chromatographic column used was a 5-µm Luna-NH_2 100A (4.6 × 250 mm) from Phenomenex and the HPLC detector was a refraction index 2410 from Waters. The mobile phase was acetonitrile/water 78/22 (v/v) at 1 mL/min. The temperature of the column was set at 30°C.

2.5. Analysis of FOS by HPAEC-PAD

Analysis of FOS composition was carried out by high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) on a Dionex ICS3000 system consisting of an SP gradient pump, an electrochemical detector with a gold working electrode and Ag/AgCl as reference electrode, and an autosampler (model AS-HV). All eluents were degassed by

flushing with helium. A pellicular anion-exchange 4 × 250 mm Carbo-Pack PA-1 column (Dionex) connected to a 4 × 50 mm CarboPac PA-1 guard column was used at 30°C. Eluent preparation was performed with Milli-Q water and NaOH. The initial mobile phase (at 0.5 mL/min) was 100 mM NaOH. A gradient from 0 to 200 mM sodium acetate was performed in 50 min at 0.5 mL/min, and 200 mM sodium acetate was maintained for 25 min. The chromatograms were analyzed using Chromeleon software. The identification of the different carbohydrates was done on the basis of standards commercially available or purified in our laboratory.

2.6. Isolation of an unknown carbohydrate

The biocatalytic reaction with the *C. cladosporioides* $CF_2$15 enzymatic preparation was scaled up to 10 mL. At the point of maximum concentration of the unknown oligosaccharide, the reaction was stopped by inactivation at 100ºC (10 min) followed by filtration. The mixture was purified by semi-preparative HPLC using a system equipped with a Waters Delta 600 pump coupled to a 5 µm Kromasil-NH_2 column (10 x 250 mm; Analisis Vinicos). A three-way flow splitter (model Accurate, Dionex) and a refraction index detector (Waters, model 2410) equilibrated at 30ºC were used. Acetonitrile/water 70:30 (v/v), degassed with helium, was used as mobile phase at 4.7 mL/min for 40 min. The column temperature was kept constant at 30ºC. After collecting the different oligosaccharides, the mobile phase was eliminated by rotary evaporation in a R-210 rotavapor (Buchi).

2.7. Mass Spectrometry

The unknown carbohydrate was analyzed by MALDI-TOF mass spectrometry (Bruker, model Ultraflex III TOF-TOF) using 2,5-dihydroxybenzoic acid doped with NaI as matrix, in positive reflector mode.

2.8. Nuclear Magnetic Resonance (NMR)

The structure of the unknown carbohydrate was elucidated using a combination of 1H, 13C and 2D-NMR (COSY, TOCSY, NOESY, HSQC, HMBC) techniques. The spectra of the sample (ca. 10 mM), dissolved in deuterated water, was recorded on a Bruker AVANCE DRX500 spectrometer equipped with a tuneable broadband 1H/X probe with a gradient in the Z axis, at a temperature of 298 K. Chemical shifts were expressed in ppm with respect to the 0 ppm point of DSS, used as internal standard. COSY, NOESY, HSQC, HSQC-TOCSY, DEPT-HSQC and HMBC sequences were provided by Bruker. COSY, TOCSY (80 ms mixing time), and NOESY (500 ms mixing time) experiments were performed with 8, 32, and 64 scans, respectively, with 256 increments in the indirect dimension and with 1024 points in the acquisition dimension. The spectral widths were 5 ppm in both dimensions. The HSQC and related experiments (16 scans) also used 256 increments in the indirect dimension and 1024 points in the acquisition dimension. The HMBC (64 scans) used 384 increments in the indirect dimension and 1024 points in the acquisition dimension. The spectral width for the heteronuclear correlations was 120 ppm in the indirect dimension and 5 ppm in the acquisition one.

3. RESULTS AND DISCUSSION

3.1. Screening of transfructosylation activity

Filamentous fungi (45 strains), isolated from sucrose-rich environments (jams and molasses), were firstly grown on a MY medium (see Experimental Section) containing 200 g/L sucrose and FOS production was followed during the growth. The carbohydrate composition of the supernatant was analyzed by HPLC-HILIC, showing that 14 strains (Table 1) were able to produce FOS with different total concentrations (referred to the total amount of sugars in the sample) and compositions (indicated in the table by the kestose/nystose ratio, K/N). Two strains ($CF_2$15 and CK1, identified as *Cladosporium cladosporioides* and *Penicillium sizovae,* respectively) gave maximum FOS production in shorter times (48 h) and were selected for their ability to synthesize FOS with different K/N ratios (0.8 and 6.0, respectively), at the point of maximum FOS concentration. Notably, *Cladosporium cladosporioides* gave the highest production of total FOS, whereas *Penicillium sizovae* was very selective, furnishing kestose as the main product.

3.2. FOS production with lyophilized mycelium of *C. cladosporioides* and *P. sizovae*

The extracellular and cell-bound transfructosylating activity of *P. sizovae* and *C. cladosporioides* to produce FOS was evaluated using cultures grown on liquid MY medium with and without 200 g/L sucrose. Cell-free supernatant (broth fraction) and washed mycelium were independently assayed to locate the transfructosylating activity. The supernatant gave conversions into total FOS lower than 5% for both

strains, showing that the activity was mostly mycelium-bound; no significant differences were observed in the activity of mycelium grown in the medium with or without sucrose. Therefore, mycelium of cultures grown in liquid MY medium without sucrose was used for next experiments aimed at optimization and product characterization. Mycelia did not lose any significant activity upon lyophilisation (data not shown). It is well reported that lyophilized mycelia of fungi are easy-to-handle biocatalysts often showing remarkable long-term stability [27,28]. Lyophilized mycelia of the two strains were used for FOS production using various initial sucrose concentrations (200-600 g/L), and the highest yields of total FOS produced were obtained with 600 g/L of sucrose (Table 2). The time course of total FOS formation with *P. sizovae* and *C. cladosporioides* using 600 g/L sucrose was followed by HPLC-HILIC and is represented in Figs. 1A and 1B, respectively.

P. sizovae produced 184 g/L of total FOS (31% w/w of total sugars, after 24 h), which was obtained at 53% sucrose conversion, whereas *C. cladosporioides* synthesized 339 g/L of FOS (56.5% w/w, after 72 h) at 93% sucrose conversion. The fact that maximum FOS concentration with *C. cladosporioides* enzyme was obtained when only 7% of initial sucrose remains in the mixture indicates that the transglycosylation to hydrolysis ratio of this enzyme is notable [29]. The FOS yield obtained with *C. cladosporioides* is close to the maximum values reported (around 60%) for the industrial processes with *Aspergillus* or *Aureobasidium* sp. enzymes [29-31].

3.3. Characterization of synthesized FOS

HPAEC-PAD was employed for the characterization of the FOS synthesized in the reactions with *P. sizovae* and *C. cladosporioides* using 600 g/L sucrose. According to the chromatograms presented in Figure 2, we detected at least 13 different carbohydrates in the reactions mediated by *P. sizovae* (Fig. 2A) and *C. cladosporioides* (Fig. 2B) mycelia. Peaks ***1***, ***2***, and ***3*** corresponded to glucose, fructose and sucrose, respectively. As illustrated in the chromatogram 2B, the main products present in the reaction mixture with *C. cladosporioides* were peaks ***4*** (1-kestose) and ***9*** (nystose). Peaks ***7***, ***8*** and ***10*** were identified as 6-kestose, neokestose and neonystose, respectively, using standards previously purified in our laboratory as described [9;13]. Peak ***12*** was the pentasaccharide ^{1}F-fructosylnystose. The oligosaccharides corresponding to peaks ***6***, ***11*** and ***13*** could not be identified so far. Figure 3 illustrates the structures of the different carbohydrates obtained in these reactions.

The compound corresponding to peak ***5*** was purified by semi-preparative HPLC. Its mass spectrum showed that it was a disaccharide. The 1D and 2D ^{1}H NMR spectra displayed two anomeric signals, arising from the typical α/β equilibrium and a signal pattern recognizable as fructose and glucose residues. From the combination of the signals from COSY, TOCSY, NOESY, HSQC and HMBC spectra, full assignment of the ^{1}H and ^{13}C resonance signals belonging to the different residues was achieved. The glycosylation position was determined from the existence of a crosspeak between the H6 from glucose and the quaternary carbon C2 from fructose in the HMBC spectrum. The NMR data unequivocally permitted to identify the compound as blastose [Fru-β(2→6)-Glc] (Figure 4), a sucrose isomer member of the

neoFOS series. To our knowledge this is the first report in which the isolation and chemical characterization of blastose is described.

3.4. Kinetics of FOS formation

The FOS formation was analyzed in detail using HPAEC-PAD. Figure 5 illustrates the profile of the biotransformation with lyophilized mycelium of *C. cladosporioides* starting from 600 g/L of sucrose. At the point of maximum FOS concentration (72 h), the FOS fraction was mainly composed of 1-kestose (158 g/L) and nystose (97 g/L), with formation of lower amounts of the disaccharide blastose (34 g/L), ^{1}F-fructosylnystose (19 g/L), 6-kestose (12 g/L) and neokestose (10 g/L). Neonystose was only slightly detected at the end of the reaction (96 h). Such a complex mixture of ^{1}F-FOS, ^{6}F-FOS and ^{6}G-FOS has only been described with the β-fructofuranosidase from *Rhodotorula dairenensis* [32].

The concentration of neokestose never surpassed 10 g/L throughout the reaction; however, blastose concentration was significantly higher (> 30 g/L) after 48 h. This result suggests that blastose is not formed by hydrolysis of neokestose, but by the transfer of fructosyl moiety to the released glucose in the medium. In fact, the biosynthetic activity detected in the *C. cladosporioides* mycelium is not very efficient to hydrolyze the β(2→6) linkages between a fructose and a glucose, as the neoFOS concentration is not diminishing throughout the process, in contrast with ^{1}F-FOS (Fig. 5). To our knowledge, this is the first report describing the formation of blastose in this kind of transfructosylating reactions.

Figure 6 shows the kinetics of FOS formation with lyophilized mycelium of *P. sizovae*. At the point of maximum FOS yield (24 h), 156 g/L out of the total FOS

concentration (184 g/L) corresponded to 1-kestose. The FOS fraction was completed with nystose (11 g/L), neokestose (6 g/L) and neonystose (11 g/L). The *P. sizovae* enzyme displays a more typical profile with major formation of ^{1}F-FOS. However, its transglycosylation to hydrolysis ratio is less favourable than that of *C. cladosporioides*. It is interesting to note the negligible presence of blastose with the *P. sizovae* enzyme, which indicates its much lower tendency to use glucose as acceptor to form β(2→6) linkages.

4. CONCLUSION

The main enzymes used for industrial production of FOS generally provide a mixture of molecules with the inulin-type structure, ^{1}F-FOS, whereas those from yeasts usually form levan-type FOS (^{6}F-FOS) or neoFOS (^{6}G-FOS). In this work, two filamentous fungi (*Cladosporium cladosporioides* and *Penicilium sizovae*) showing mycelium-bound transfructosylating activity were isolated. Maximum FOS yields were 56% and 31% for *C. cladosporioides* and *P. sizovae* respectively. Interestingly, *C. cladosporioides* synthesized a mixture of ^{1}F-FOS, ^{6}F-FOS and ^{6}G-FOS, including the presence of a non-conventional disaccharide (blastose). Considering that the FOS yield with *C. cladosporioides* is close to that obtained with typical *Aspergillus* or *Aureobasidium* enzymes, the formation of a mixture of FOS with different glycosidic linkages could give rise to certain benefits regarding their bioactivity.

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Figure legends

Figure 1. Time course of the reaction of sucrose with *P. sizovae* **(A)** and *C. cladosporioides* **(B)** mycelia. Reaction conditions: 600 g/L sucrose, 40 g/L lyophilized mycelium, 20 mM sodium acetate buffer (pH 6.0), 50ºC.

Figure 2. HPAEC-PAD analysis of the reaction of sucrose with *P. sizovae* **(A)** and *C. cladosporioides* **(B)** mycelia at the point of maximum FOS concentration. Peaks: ***1***: glucose; ***2***: fructose; ***3***: sucrose; ***4***: 1-kestose; ***5***: blastose; ***7***: 6-Kestose; ***8***: neokestose; ***9***: nystose; ***10***: neo-nystose; ***12***: ^{1}F-fructosylnystose; ***6***, ***11***, ***13***: unknown.

Figure 3. Structure of the fructooligosaccharides produced by *P. sizovae* and *C. cladosporioides* transfructosylating activity.

Figure 4. 2D-NMR DEPT-HSQC spectra of blastose [Fru-β(2→6)-Glc]. The signals are assigned and labelled. The key points for identifications are also shown.

Figure 5. Kinetics of FOS formation using 600 g/L sucrose catalyzed by lyophilized *C. cladosporioides*. Reaction conditions: 40 g/L lyophilized mycelium, 20 mM sodium acetate buffer (pH 6.0), 50ºC.

Figure 6. Kinetics of FOS formation using 600 g/L sucrose catalyzed by lyophilized *P. sizovae* (40 g/L). Reaction conditions: 40 g/L lyophilized mycelium, 20 mM sodium acetate buffer (pH 6.0), 50ºC.

Table 1. Initial screening of FOS-synthesizing microorganisms in fruit jams and molasses. Experimental conditions: biotransformations with growing cells in MY medium (including 200 g/L sucrose), 28ºC, 150 rpm. Carbohydrates: F, fructose; G, glucose; S, sucrose; K, total kestoses; N, total nystoses; FN fructosylnystose; K/N, kestose/nystose ratio.

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Strain	**Time (h)[a]**	**Composition (%) [b]**						**K/N**
		F (%)	**G (%)**	**S (%)**	**K (%)**	**N (%)**	**FN (%)**	**ratio**
$CF_2$3V	96	2	33	9	32	24	-	1.3
$CF_2$4V	96	8	43	9	12	18	9	0.7
$CF_2$15	48	5	34	6	23	27	5	0.8
CK1	48	12	25	35	24	4	-	6.0
M1A	96	4	30	13	36	17	-	2.1
$CF_1$1	72	2	18	50	21	9		4,4
$CF_1$2	48	11	14	69	3	-	-	
$CF_2$7	96	3	12	67	13	7	-	
$CF_2$9V	48	48	47	3	3	-	-	-
$CF_2$11	72	3	5	87	4	-	-	-
$CF_2$12	96	8	20	57	10	5	-	2.0
$CF_2$14	96	6	31	43	18	4	-	4.0
$CF_2$16	48	2	24	47	12	5	-	2.4
SD4	96	5	26	58	16	5	-	3.2

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[a] Time of maximum FOS production

[b] Weight percentage referred to the total amount of sugars in the mixture

Table 2. FOS production using lyophilised mycelium of *Cladosporium cladosporioides* and *Penicilium sizovae* using different substrate concentrations.

Strain	[S] (g/L)	Time (h)[a]	Composition (%) [b]					
			F (%)	G (%)	S (%)	K (%)	N (%)	FN (%)
C. cladosporioides	200	24	8	30	10	23	7	7
C. cladosporioides	400	48	4	30	18	32	11	5
C. cladosporioides	600	72	3	33	12	30	18	4
P. sizovae	200	24	19	30	25	24	2	-
P. sizovae	400	24	12	23	36	25	4	-
P. sizovae	600	24	4	17	47	27	5	-

[a] Time of maximum FOS production

[b] Weight percentage referred to the total amount of sugars in the mixture